Expression of Human Immunodeficiency Virus Type 1 Neutralizing Antibody Fragments Using Human Vaginal *Lactobacillus*

Angela Marcobal,¹ Xiaowen Liu,¹ Wenlei Zhang,² Antony S. Dimitrov,² Letong Jia,¹ Peter P. Lee,¹ Timothy R. Fouts,² Thomas P. Parks,¹ and Laurel A. Lagenaur¹

Abstract

Eradication of human immunodeficiency virus type 1 (HIV-1) by vaccination with epitopes that produce broadly neutralizing antibodies is the ultimate goal for HIV prevention. However, generating appropriate immune responses has proven difficult. Expression of broadly neutralizing antibodies by vaginal colonizing lactobacilli provides an approach to passively target these antibodies to the mucosa. We tested the feasibility of expressing single-chain and single-domain antibodies (dAbs) in *Lactobacillus* to be used as a topical microbicide/live biotherapeutic. Lactobacilli provide an excellent platform to express anti-HIV proteins. Broadly neutralizing antibodies have been identified against epitopes on the HIV-1 envelope and have been made into active antibody fragments. We tested single-chain variable fragment m9 and dAb-m36 and its derivative m36.4 as prototype antibodies. We cloned and expressed the antibody fragments m9, m36, and m36.4 in *Lactobacillus jensenii*-1153 and tested the expression levels and functionality. We made a recombinant *L. jensenii* 1153-1128 that expresses dAb-m36.4. All antibody fragments m9, m36, and m36.4 were expressed by lactobacilli. However, we noted the smaller m36/m36.4 were expressed to higher levels, $\geq 3 \mu\text{g/ml}$. All *L. jensenii*-expressed antibody fragments bound to gp120/CD4 complex; *Lactobacillus*-produced m36.4 inhibited HIV-1_{BaL} in a neutralization assay. Using a TZM-bl assay, we characterized the breadth of neutralization of the m36.4. Delivery of dAbs by *Lactobacillus* could provide passive transfer of these antibodies to the mucosa and longevity at the site of HIV-1 transmission.

Keywords: HIV neutralization, single-domain antibody, *Lactobacillus*, passive delivery

Introduction

NEUTRALIZING ANTIBODIES WERE among the first inhibitors identified against human immunodeficiency virus type 1 (HIV-1) infections. Active immunization to generate an appropriate *de novo* HIV neutralizing antibody response remains the ultimate goal but has proved challenging.¹ Passive topical delivery of antiviral antibodies at or on mucosal surfaces, in the vaginal and cervix, can interrupt the cycle of virus infection^{2–6}; however, obtaining and maintaining effective levels of such antiviral antibodies can be costly and short lived.

Thus, new strategies are needed for providing neutralizing antibodies at the mucus surface where HIV is transmitted. One innovative avenue is to use a recombinant commensal *Lacto-*

bacillus as a vehicle for releasing neutralizing antibodies at the mucosa. *Lactobacillus* species are common members of the vaginal microbiota in healthy women of childbearing age^{7,8} and interact with the host to reduce proinflammatory cytokine levels and serve as the major host inflammatory modulator.⁹ *Lactobacillus jensenii* is one of the more predominant vaginal lactobacilli. The strain *L. jensenii* 1153 has been proven as a potential platform to deliver sufficient levels of the anti-HIV protein-modified cyanovirin-N to mucosal surfaces to protect macaques from Simian (S)HIV challenge.¹⁰

HIV-1 entry into target cells requires the sequential binding of the viral envelope glycoprotein gp120 epitopes to CD4 and then to the CCR5 chemokine receptor.¹¹ When CD4 binds to the HIV envelope, a conformation change in gp120 exposes the cryptic epitopes known as the CD4-induced

¹Osel, Inc., Mountain View, California.

²Profectus Biosciences, Inc., Baltimore, Maryland.

(CD4i) site.^{12,13} When a CD4i antibody is present, it recognizes these previously unexposed sites, blocking virus attachment of HIV to the chemokine receptor.¹⁴

Different variants of anti-HIV antibodies have been developed, such as single-chain variable fragments (scFvs), small antibody fragments, and even smaller domain antibodies (dAbs). These alternatives offer advantages over conventional antibodies because of their smaller size, robustness, and their ability to be expressed in bacteria. Moreover, the small size of the dAbs and scFvs overcomes the steric constraints when the virion is bound to CD4; an intact IgG molecule may not be able to access the CD4i bridging sheet.¹⁵ Numerous scFvs and dAb variants and camel-derived antibodies have now been identified that retain the broadly neutralizing anti-HIV activity.^{16–22} Among these broadly neutralizing antibody fragments are ScFv-m9,^{23,24} dAb-m36,^{25,26} and dAb-36.4 variant¹⁹ that bind CD4i regions and facilitate neutralization of a broad range of HIV isolates.

We selected these antibody fragments that target the highly conserved CD4i epitopes, for expression in *Lactobacillus* as entry inhibitors, since they are likely to be present across the wide genetic diversity of HIV-1 variants. In addition, their small size may put less strain on the recombinant *Lactobacillus* allowing for expression levels that are biologically relevant.

In this article, we optimized and compared the expression of scFV-m9, dAb-m36, and dAb-m36.4 by *L. jensenii*. We evaluated the breadth and potency of dAb-m36.4 against different HIV-1 subtypes. Finally, we constructed a recombinant *L. jensenii* 1153-1128 producing dAb AP-m36.4.

Materials and Methods

Bacterial strains, culture, plasmid construction, and transformation

L. jensenii strain 1153, a vaginal isolate from a healthy woman, was cultivated as described previously.²⁷ To express m9 or m36 antibody fragments in *L. jensenii* 1153, the m9 or m36 genes were cloned into the modular shuttle vector pOSEL175, a modified version of pOSEL144.²⁷ The expression cassette for secreted scFv-m9 or dAb-m36 contained the *Lactococcus* promoter p23, the signal sequence from the ribosome binding site to the signal peptidase cleavage site of the *L. crispatus* S-layer gene (*CbsAss*) (for protein secretion), and a C-terminal epitope tag DYKDDDDK (FLAG) with a TAA stop codon at the 3' end.

For purification ease, Profectus Biosciences Inc. modified the cassettes for the m9, m36, and m36.4 genes to include a C-terminal albumin-binding domain (ABD), SA06 for improving its pharmacokinetic properties and also aiding in purification using rat serum albumin (RSA) columns.²⁸ The original m9 and m36 genes do not bind albumin. The OSEL shuttle plasmids were maintained and propagated in *Escherichia coli*, purified, and electroporated into *L. jensenii* 1153 as described before,²⁷ generating the transformed strains *L. jensenii* 1153-1126p (expressing m9), *L. jensenii* 1153-1127p (expressing m36), and *L. jensenii* 1153-1128p (expressing m36.4). Transformed lactobacilli were routinely propagated using Man Rogosa Sharpe (MRS) or Rogosa media (broth or agar) with 20 µg/ml of erythromycin. *L. jensenii* 1153-1128 was propagated on MRS or Rogosa broth or agar without antibiotic.

Western blots

Stationary-phase supernatants from transformed (plasmid expressed) *L. jensenii* 1153-1126p, *L. jensenii* 1153-1127p, and *L. jensenii* 1153-1128p were harvested and centrifuged to remove cell debris. Thirty microliters of supernatant was run on a 12% Bis-Tris Gel (Life Technologies) and transferred to polyvinylidene difluoride membranes using an iBlot (Life Technologies). The m9, m36, or AP-m36.4 proteins were detected using monoclonal mouse anti-FLAG antibody (Pierce Thermo) dilutes 1:1,000 and the anti-mouse Western Breeze Chemiluminescent Kit (Life Technologies).

Purification and bioactivity characterizations of *Lactobacillus*-derived m36.4

The *L. jensenii* strains expressing m9 and m36 (each containing the ABD) from plasmids were cultured in Rogosa SL broth (Difco) to stationary phase. Cell-free supernatants were centrifuged, filtered, and purified using the (i) albumin Sepharose column (RSA bound to cyanogen bromide [CNBr]-activated Sepharose 4B; GE Healthcare) or (ii) full-length single-chain (FLSC) column (gp120/CD4 complex bound to CNBr-activated Sepharose 4B). In both methods, the columns were equilibrated with phosphate-buffered saline (PBS), the supernatant supplemented with 0.5 M sodium chloride (NaCl) passed slowly through the column to ensure at least 1 min of contact time, column washed with at least 20 bed volumes PBS supplemented with 0.5 M NaCl and m36 eluted with 0.2 N citric acid, pH 3.0.

The eluant was immediately neutralized to pH 6.0–8.0 using 1 M Tris at pH 9.0 and dialyzed against PBS. Control *E. coli* expressed m36.4 was purified by sequential ion exchange column (Q HP Sepharose Fast Flow columns; GE Healthcare Life Sciences) and size exclusion column (Superdex 75 column, 120 ml bed volume; GE Healthcare). Periplasmic extract from HB2151 expressing m36.4 was dialyzed against 20 mM piperazine, pH 5.8, centrifuged at 14,000 g for 30 min, and loaded onto the Q HP column. The column was washed with 20 mM piperazine, 0.15 M NaCl, pH 5.8 buffer, and the bound protein eluted using gradient salt elution from 0.15 to 0.50 M NaCl. M36 and m36.4 eluted in the peak in the range of 0.2 M NaCl. The collected eluant from this peak was passed through a size exclusion column to eliminate the majority of high-molecular weight contaminants.

Preparations of m9, m36, and m36.4 were quantified by Coomassie blue staining and gp120/CD4 capture enzyme-linked immunosorbent assay (ELISA). FLSC (CD4-gp120) leached from column was removed by an additional passage over an RSA column. The amount of RSA leached from the column was insignificant.

Although LPS levels were not measured following purification from *E. coli*, similar antibody fragments purified previously had <10 EU/mg of protein.

Gp120/CD4 binding (capture ELISA)

A sheep antibody, D7324 (Aalto Bio Reagents), which binds to a motif (amino acid 497–511) in the envelope gene gp120, was coated on ELISA plates at 2°C–8°C, at a concentration of 2 µg/ml, overnight. Plates were then washed with Tris-buffered saline with Tween (TBST) and blocked with 5% skim milk. FLSC (a gp120/CD4 complex), at a

concentration of 0.2 $\mu\text{g}/\text{ml}$ in PBS, was captured on D7324 and the plate was washed with TBST. Titrated samples of m9, m36, and m36.4 solution were overlaid onto the FLSC, incubated for 1 h, and washed with TBST. All antibody fragments bound to FLSC and were detected using the horseradish peroxidase (HRP)-conjugated mouse anti-FLAG antibody (clone M2; Sigma).

Peripheral blood mononuclear cell neutralization assay (p24 assay)

Human peripheral blood mononuclear cells (PBMCs) were purchased from SeraCare Life Sciences, Inc. HIV-1_{BaL} virus was initially received from the NIH AIDS Reagents Program and later grown by Profectus Biosciences for laboratory use. PBMCs were infected with HIV-1_{BaL} at a multiplicity of infection of 0.01 in the presence of m36.4 (purified as described above) with desired concentrations. The mixture was incubated at 37°C for 1 week, and the supernatant was lysed using 1% Triton X-100 and quantified for p24 presence by using a p24 capture ELISA developed at Profectus Biosciences, Inc. Plates were coated with 3 $\mu\text{g}/\text{ml}$ mouse monoclonal anti-p24 antibody (clone 39/6.14 by ZeptoMetrix) washed with TBST, blocked with 5% casein and 4% rabbit serum, washed with TBST, overlaid with the lysed p24 containing supernatant, incubated for 1 h, and washed with TBST. Bound p24 was detected using the HRP-conjugated rabbit polyclonal anti-p24 antibody (1303-P; ImmunoDX, LLC).

Construction of recombinant *L. jensenii* 1153-1128

Before insertion of the antibody cassette into the *Lactobacillus* chromosome, two modifications were made. The two-amino-acid peptide (AP), corresponding to the N terminus of the mature CbsA protein, was inserted downstream of the CbsA signal sequence at the N-terminus of the m36 protein to more closely resemble the native signal peptidase cleavage site of this protein. A point mutation at amino

acid 44 glutamine (Q) to glutamic acid (E) Q44E was substituted for the original sequence.¹⁹ This point mutation, also called m36.4, confers greater breadth against HIV-1. The AP-m36.4 gene was cloned into Osel's integration vector.

For stable expression of AP-m36.4 in *Lactobacillus*, the AP-m36.4 gene expression cassette was integrated in single copy into the *L. jensenii* 1153 genome by homologous recombination into the *minor capsid* gene. The recombinant strain was selected by replicate plating into MRS agar with and without erythromycin. During successful integration, resolution of the plasmid occurs, which removes the erythromycin resistance gene. The integrated and resolved strain *L. jensenii* 1153-1128 contains a single copy of AP-m36.4 gene and requires no antibiotic selection. The recombinant strain *L. jensenii* 1153-1128 was maintained in the MRS broth.

TZM-bl neutralization assay

The TZM-bl neutralization assay was used to evaluate antibody breadth and potency of m36.4.²⁹ Neutralization experiments are expressed as IC₅₀ titer of the protein in $\mu\text{g}/\text{ml}$. m9 and m36 were not tested.

Results

Expression of antibody fragments in *L. jensenii*

We tested the feasibility of expressing anti-HIV-1 antibody fragments using a human vaginal *Lactobacillus* expression platform to produce a recombinant live biotherapeutic against HIV-1. We examined the scFv-m9^{23,24} and single dAb-m36 (CD4i epitope)^{25,26} and its derivative m36.4.¹⁹ We cloned the m9 and m36 genes, which contained an ABD for purification²⁸ and FLAG tag (for easy detection) into Osel's *Lactobacillus* expression vector p175 under the p23 promoter and transformed *L. jensenii* 1153. A diagram of the plasmid construction of pOSELp23-m9 and pOSELp23-m36 is shown (Fig. 1A). Supernatants from *L. jensenii* secreting m9 or m36 are detected by using an anti-Flag antibody and

FIG. 1. Plasmids used in this study and protein expression. (A) Expression plasmids for scFv-m9 and dAb-m36 are shown. Plasmids contain promoter P23 (green), Lcsp (red), antibody fragment (blue), ABD (pink), and Flag epitope (orange). (B) Proteins and standards were run on 12%-Bis-Tris gels, transferred to PVDF membrane, and visualized with anti-FLAG antisera. Lane 1, m9 standard 20 ng, Lane 2, *Lactobacillus jensenii* expressed m9 (30 μl loaded), Lane 3, m36 standard 20 ng, Lane 4, m36 standard 10 ng, Lane 5, *L. jensenii* expressed m36 (10 μl loaded). ABD, albumin-binding domain; dAb, domain antibody; Lcsp, *L. crispatus* signal sequence; PVDF, polyvinylidene difluoride; scFv, single-chain variable fragment.

compared with a purified protein standard produced in *E. coli* (Fig. 1B). The estimated concentrations of m9 and m36 secreted were ~ 660 and $4.0 \mu\text{g/ml}$, respectively.

Binding of antibody fragments to FLSC

Supernatants of scFv-m9 and m36 expressed from plasmids transformed into *L. jensenii* 1153 were purified and tested for functionality by determining binding to FLSC (Fig. 2A, B). The scFv-m9 plasmid expressed by *L. jensenii* or scFv-m9 expressed by *E. coli* bound to the gp120-CD4 complex in ELISA, while the control supernatant from *L.*

jensenii did not (data not shown); however, scFv-m9 expressed by *L. jensenii* bound with lower affinity (Fig. 2A). Due to lower expression levels and lower affinity, scFv-m9 was not pursued further.

L. jensenii plasmid-expressed m36 bound to FLSC to the same level as the standard (Fig. 2B). The recombinant *L. jensenii* 1153-1128 expressed a modified version of m36.4, with the addition of amino acids alanine (A) and proline (P) at the amino terminus. Addition of the AP-sequence was previously shown to improve the secretion of full-length protein from *L. jensenii*.³⁰ Both *E. coli*-expressed m36.4 and recombinant *L. jensenii* 1153-1128-expressed AP-m36.4 were tested to confirm that the addition of AP to the amino terminus of m36.4 did not influence binding to FLSC (Fig. 2C). The EC₅₀s were comparable: *E. coli* produced dAb-m36.4 12.7 ng/ml and recombinant *L. jensenii* 1153-1128 produced AP-m36.4 11.9 ng/ml (no statistical difference).

Antiviral activity of m36.4

L. jensenii-expressed m36.4 was purified using two techniques; purity of the protein using the different strategies is shown in Figure 3A. Purified m36.4 was tested for anti-HIV activity using a p24-PBMC-based assay (Fig. 3B). Both RSA-purified m36.4 and FLSC-purified m36.4 showed similar reduction of p24, and thus, the *Lactobacillus*-produced m36.4 protein was active against HIV_{BaL} with an IC₅₀ of $\sim 1 \mu\text{g/ml}$.

Neutralization breadth of m36.4

Table 1 details the breadth of neutralization of the m36.4 protein. m36.4 was purified from *E. coli* and tested for activity against a panel of 68 pseudoviruses from each of the major genetic subtypes representing clades A, B, C, D, G, including transmitted founders (T/F) and circulating recombinant forms CRF01 AE and CRF02 AG. Table 1 shows the broadly neutralizing activity of m36.4 against clades B, C, and D; most IC₅₀ values were in the $\leq 5 \mu\text{g/ml}$. Less or no activity was noted against clades A, G, and CRFs. Previously, we tested a partially purified supernatant from wild-type *L. jensenii* in pseudovirus assays with HIV-1_{HXB2} envelope and found no inhibition.²⁷ In addition, m36.4 did not inhibit MuLV control in the TZM-bl assay.

Construct integration vectors and integrated single domain expression cassettes into the *L. jensenii* 1153 chromosome and tested phenotypic stability

We constructed a recombinant *L. jensenii* 1153 by integrating an optimized AP-m36.4 antibody expression cassette into the *minor capsid* gene of *L. jensenii* 1153 through homologous recombination (Fig. 4A). The strains generated were evaluated for complete resolution of the plasmid and antibody production (not shown). A diagram of the two sets of internal and external primers, which were used to amplify the integration region within the *minor capsid* gene, is shown (Fig. 4B).

Polymerase chain reaction (PCR)-amplified genomic DNA from the parental *L. jensenii* 1153 and recombinant strain designated *L. jensenii* 1153-1128 were run on a 2% agarose gel. *L. jensenii* 1153 shows a 353 bp band corresponding to a region of the *minor capsid* gene and a 1,824 bp band of the entire *minor capsid* gene (Fig. 4B). *L. jensenii*

FIG. 2. ELISA readout of binding of antibody fragments to FLSC (gp120/CD4) and purity of m36.4 and AP-m36.4. Comparison of binding properties from antibody fragments produced by *L. jensenii* (blue) to *Escherichia coli* produced standards (red). (A) scFv-m9; produced from plasmid (B) dAb-m36; produced from plasmid (C) dAb-m36.4 (*E. coli*) versus dAb-AP-m36.4 (recombinant *L. jensenii* 1153-1128). Alanine proline N-terminal m36.4 (AP-m36.4). ELISA, enzyme-linked immunosorbent assay; FLSC, full-length single chain.

FIG. 3. Purification of AP-m36.4 protein used for HIV inhibition assay. (A) Lane 1: see Blue Plus Prestained ladder (Invitrogen), Lane 2: m36.4 *E. coli*, 3 µg (purified on Q, HP, size-exclusion columns), Lane 3: *L. jensenii* 1153-1128 expressed AP-m36.4, 3 µg (purified on RSA column), Lane 4: *L. jensenii* 1153-1128 expressed AP-m36.4, 7.4 µg total protein (purified on FLSC column) * FLSC (CD4-gp120) leached from column was removed by RSA. (B) AP-m36.4 produced by recombinant *L. jensenii* 1153-1128 inhibits HIV_{BAL}. Bar graph showing the p24 value measured. Each value is average of four replicates. IC₅₀ for AP-m36.4 produced in *Lactobacillus* is ~1 µg/ml. RSA, rat serum albumin.

1153-1128 shows a 1,250 bp band corresponding to a region of the *minor capsid* gene with the 891 bp band (+6 bp restriction site) cassette insertion. The external primers amplify a 2,721 bp band (1,824 + 891 + 6 bp).

The strain was passaged 40 generations, genomic DNA was extracted, and the cassette was sequenced (not shown). The 40-generation sample was also analyzed by PCR for stability. No changes were noted in the size of the internal and external primer bands 891 and 2,721, respectively, and thus, the *L. jensenii* 1153-1128 recombinant is genotypically stable. We examined phenotypic stability by performing a carbohy-

drate utilization profile of parental strain *L. jensenii* 1153. Both *L. jensenii* 1153 and *L. jensenii* 1153-1128 fermented glucose, D-fructose, D-mannose, N-acetylglucosamine, amygdalin, arbutin, wells, salicin, D-cellobiose, D-maltose, D-saccharose, amidon (starch), and gentiobiose wells and hydrolyze esculin ferric citrate (data not show).

We tested growth of the 1153-1128 strain and lactic acid production (pH) and compared them to parental strain *L. jensenii* 1153. *L. jensenii* 1153-1128 grew to the same level as the parental strain and reduced pH of the media under aerobic (5% CO₂) and anaerobic growth conditions (Fig. 4C, D).

TABLE 1. IC₅₀ TITER M36.4 IN TZM-BL CELLS (µG/ML)

A		BC		CRF01-AE	
Q461.e2	3.433	CNE19	0.446	620345.c01	>20
Q769.d22	2.759	CNE20	0.757	R2184.c04	>20
Q23.17	6.507	CNE17	1.357	R1166.c01	>20
Q259.d2.17	11.80	CNE30	6.113	R3265.c06	>20
0330.v4.c3	8.322	CNE52	0.508	C3347.c11	>20
0260.v5.c36	19.80	CNE53	3.008	C4118.c09	>20
AC		C		CNE5	>20
3301.v1.c24	1.464	Du156.12	3.729	CRF01-AG	
6041.v3.c23	3.521	Du172.17	1.926	T250-4	0.694
6540.v4.c1	>20	Du422.1	2.904	T255-34	0.814
ACD		ZM197M.PB7	0.937	T257-31	11.71
0815.v3.c3	0.492	ZM233M.PB6	10.77	928-28	16.68
3103.v3.c10	2.763	ZM249M.PL1	0.210	263-8	8.705
B		ZM53M.PB12	0.286	T251-18	>20
6535.3	0.949	ZM214M.PL15	1.876	T278-50	11.46
QH0692.42	0.965	C (T/F)		D	
SC422661.8	1.400	Ce0393_C3	0.262	3016.v5.c45	0.377
PVO.4	0.810	Ce1176_A3	2.650	A07412M1.vrc12	0.953
TRO.11	3.666	Ce2010_F5	3.621	231965.c01	3.720
AC10.029	1.260	BF1266.431a	0.256	D (T/F)	
RHPA4259.7	3.885	CD		191821_E6_1	0.194
THRO4156.18	2.059	3817.v2.c59	3.484	G	
B (T/C)		6952.v1.c20	0.734	X1193_c1	>20
WEAU_d15_410_5017	3.584	89-F1_2_25	0.134	P0402_c2_11	>20
1006_11_C3_1601	0.885	6811.v7.c18	>20	X1254_c3	>20
1054_07_TC4_1499	1.894			X2088_c9	>20
				X2131_C1_B5	>20
				P1981_C5_3	18.45

T/F, transmitted founders.

FIG. 4. Construction and characterization of recombinant *Lactobacillus*. **(A)** Construction of *L. jensenii* 1153-1128 was performed by site-specific integration of the p23-AP-m36.4 gene into the *minor capsid* gene locus in the *Lactobacillus* chromosome. A diagram of the recombinant construction is shown. **(B)** PCR fragment length analysis of the parent and recombinants. PCR was carried out using primers within the *minor capsid* gene and flanking the AP-m36.4 expression cassette 891 bp Int, and with primers external to the *minor capsid* gene. The parental strain shows a 353 bp fragment when amplified by internal primers and a 1,824 bp fragment when amplified by the Ext primers. Recombinant strain WCB 1128 shows a 1,250 and 2,721 bp fragment, respectively, with the insertion of the 891 cassette plus a 6 bp restriction site. A long-term (40 generation) culture also showed the same PCR fragment length pattern. **(C)** Growth curves are shown for the parental *L. jensenii* 1153 strain and recombinant *L. jensenii* 1153-1128 strain, under anaerobic and 5% CO₂ growth conditions. **(D)** Lactic acid production as measured by pH is shown for the parental *L. jensenii* 1153 strain and recombinant *L. jensenii* 1153-1128 strain, under anaerobic and 5% CO₂ growth conditions. Ext, external; Int, integration site; PCR, polymerase chain reaction; WCB, working cell bank.

Discussion

There is a tremendous need for approaches to combat HIV-1 transmission in women of reproductive age. Lactobacilli (including *L. jensenii*) are important members of the vaginal microbiome, lowering vaginal pH,^{31,32} modulating cervicovaginal inflammation,⁹ and providing resistance to HIV infection.³³ We established the feasibility of expressing bioactive anti-HIV-1 antibody fragments from a vaginal strain of *L. jensenii* and show that these fragments are functional and can be stably expressed from a recombinant *L. jensenii*. In addition, we confirmed that the recombinant *L. jensenii* 1153-1128 expressing m36.4 could colonize the rhesus macaque vagina (data not shown).

ScFv-m9 was expressed at lower levels and had lower affinity for FLSC, so it was not pursued. However, the smaller dAbs m36 and m36.4 could be expressed at high levels and thus were more viable for this approach. It is noteworthy to mention that the small dAb fragments m36 and m36.4 can bind the CD4i epitope even without the conformational change that occurs with CD4 binding to gp120.

Previously, several groups have used other commensals to express functional antibody fragments. Early work used the commensal *Streptococcus gordonii* engineered to produce

scFv H6 and an anti-idiotypic scFv with anti-*Candida* activity.^{34,35} *S. gordonii* was also used to produce Guy's 13 scFv against the caries-producing bacterium *Streptococcus mutans*.³⁶

Marcotte *et al.* expressed scFv on the surface of *Lactobacillus paracasei* against *Porphyromonas gingivalis*,³⁷ and Kruger *et al.* used *Lactobacillus casei* expressing an scFv against *S. mutans*,³⁸ both approaches reduced dental caries. *L. paracasei* was used to express an scFv to protect against anthrax edema toxin caused by the bacterium *Bacillus anthracis*.³⁹ *L. casei*, *L. paracasei*, and *Lactobacillus rhamnosus* GG were engineered to express llama VHH antibody fragments against rotavirus.^{40,41} Most recently, Andersen *et al.* described the use of single dAbs to neutralize *Clostridium difficile* toxin B.⁴²

Another noteworthy approach is the use of adeno-associated virus (AAV) to deliver broadly neutralizing antibodies.⁴³ While the approach appears to be safe, and the AAV are long lived in the tissue and can secrete antibody persistently, one caveat with this approach is the inability to remove the AAV vector easily, should the need arise. Commensal bacteria (expressing antibodies) can be removed by treatment with an appropriate antibiotic.

Although the idea of expressing antibody fragments in commensals is not new, with the increasing development of antibiotic and antiviral resistance and therefore fewer treatment options for both bacterial and viral infections, new approaches are needed. With a better appreciation of the health benefits provided by commensals as part of the microbiome, perhaps this is an approach whose time has come.

For prevention of HIV, the benefit to using human colonizing vaginal strains would allow for delivery of antibodies mucosally at the site where the virus first enters the body. Development of broadly neutralizing antibodies in infected individuals, in the blood or at mucosal sites, takes months to years. Current vaccine strategies do not uniformly generate these antibodies. Our results indicate that stable expression of anti-HIV-1 antibody fragments is possible using a common *Lactobacillus* in the vaginal microbiota as a recombinant live biotherapeutic. m36.4 was used as a prototype antibody fragment, but other newly discovered broadly neutralizing antibodies might also be candidates to test in this approach.

Acknowledgments

We thank the Michael Seamen Laboratory at Harvard Medical School for performing the TZM-bl pseudovirus assays. This work was funded, in part, by the Bill and Melinda Gates Foundation GCE Phase 1 and 2 OPP1035271 and NIH grant 5R44AI100753-03. This work was supported by the National Institutes of Health, SBIR grant 1R43AI100753-01.

Author Disclosure Statement

L.A.L., P.P.L., and T.P.P. work for Osel, a biotherapeutics company that is developing *L. jensenii* as a delivery system for anti-HIV therapeutics, and are shareholders. A.M. works for Osel. X.L. and L.J. previously worked for Osel. X.L. is a shareholder of Osel, Inc. A.S.D. and W.Z. declare no conflicts. T.R.F. is a founder and shareholder of Profectus Biosciences, Inc.

References

1. Wilson NA, Watkins DI: Is an HIV vaccine possible? *Braz J Infect Dis* 2009;13:304–310.
2. Baba TW, Liska V, Hofmann-Lehmann R, *et al.*: Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. *Nat Med* 2000;6:200–206.
3. Conley AJ, Kessler JA 2nd, Boots LJ, *et al.*: The consequence of passive administration of an anti-human immunodeficiency virus type 1 neutralizing monoclonal antibody before challenge of chimpanzees with a primary virus isolate. *J Virol* 1996;70:6751–6758.
4. Foresman L, Jia F, Li Z, *et al.*: Neutralizing antibodies administered before, but not after, virulent SHIV prevent infection in macaques. *AIDS Res Hum Retroviruses* 1998;14:1035–1043.
5. Hessel AJ, Poignard P, Hunter M, *et al.*: Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques. *Nat Med* 2009;15:951–954.
6. Hessel AJ, Rakasz EG, Poignard P, *et al.*: Broadly neutralizing human anti-HIV antibody 2G12 is effective in protection against mucosal SHIV challenge even at low serum neutralizing titers. *PLoS Pathog* 2009;5:e1000433.
7. Antonio MA, Hawes SE, Hillier SL: The identification of vaginal *Lactobacillus* species and the demographic and microbiologic characteristics of women colonized by these species. *J Infect Dis* 1999;180:1950–1956.
8. Ravel J, Gajer P, Abdo Z, *et al.*: Vaginal microbiome of reproductive-age women. *Proc Natl Acad Sci U S A* 2011;108(Suppl 1):4680–4687.
9. Anahtar MN, Byrne EH, Doherty KE, *et al.*: Cervicovaginal bacteria are a major modulator of host inflammatory responses in the female genital tract. *Immunity* 2015;42:965–976.
10. Lagenaur LA, Sanders-Beer BE, Brichacek B, *et al.*: Prevention of vaginal SHIV transmission in macaques by a live recombinant *Lactobacillus*. *Mucosal Immunol* 2011;4:648–657.
11. Berger EA, Murphy PM, Farber JM: Chemokine receptors as HIV-1 coreceptors: Roles in viral entry, tropism, and disease. *Annu Rev Immunol* 1999;17:657–700.
12. Wyatt R, Kwong PD, Desjardins E, *et al.*: The antigenic structure of the HIV gp120 envelope glycoprotein. *Nature* 1998;393:705–711.
13. Salzwedel K, Smith ED, Dey B, Berger EA: Sequential CD4-coreceptor interactions in human immunodeficiency virus type 1 Env function: Soluble CD4 activates Env for coreceptor-dependent fusion and reveals blocking activities of antibodies against cryptic conserved epitopes on gp120. *J Virol* 2000;74:326–333.
14. Xiang SH, Doka N, Choudhary RK, Sodroski J, Robinson JE: Characterization of CD4-induced epitopes on the HIV type 1 gp120 envelope glycoprotein recognized by neutralizing human monoclonal antibodies. *AIDS Res Hum Retroviruses* 2002;18:1207–1217.
15. Labrijn AF, Poignard P, Raja A, *et al.*: Access of antibody molecules to the conserved coreceptor binding site on glycoprotein gp120 is sterically restricted on primary human immunodeficiency virus type 1. *J Virol* 2003;77:10557–10565.
16. Wang HW, Cole D, Jiang WZ, *et al.*: Engineering and functional evaluation of a single-chain antibody against HIV-1 external glycoprotein gp120. *Clin Exp Immunol* 2005;141:72–80.
17. Chancey CJ, Khanna KV, Seegers JF, *et al.*: Lactobacilli-expressed single-chain variable fragment (scFv) specific for intercellular adhesion molecule 1 (ICAM-1) blocks cell-associated HIV-1 transmission across a cervical epithelial monolayer. *J Immunol* 2006;176:5627–5636.
18. Forsman A, Beirnaert E, Aasa-Chapman MM, *et al.*: Llama antibody fragments with cross-subtype human immunodeficiency virus type 1 (HIV-1)-neutralizing properties and high affinity for HIV-1 gp120. *J Virol* 2008;82:12069–12081.
19. Chen W, Xiao X, Wang Y, Zhu Z, Dimitrov DS: Bifunctional fusion proteins of the human engineered antibody domain m36 with human soluble CD4 are potent inhibitors of diverse HIV-1 isolates. *Antiviral Res* 2010;88:107–115.
20. Koh WW, Steffensen S, Gonzalez-Pajuelo M, *et al.*: Generation of a family-specific phage library of llama single chain antibody fragments that neutralize HIV-1. *J Biol Chem* 2010;285:19116–19124.
21. McCoy LE, Quigley AF, Strokappe NM, *et al.*: Potent and broad neutralization of HIV-1 by a llama antibody elicited by immunization. *J Exp Med* 2012;209:1091–1103.
22. Lutje Hulsik D, Liu YY, Strokappe NM, *et al.*: A gp41 MPER-specific llama VHH requires a hydrophobic CDR3 for neutralization but not for antigen recognition. *PLoS Pathog* 2013;9:e1003202.

23. Zhang MY, Shu Y, Rudolph D, *et al.*: Improved breadth and potency of an HIV-1-neutralizing human single-chain antibody by random mutagenesis and sequential antigen panning. *J Mol Biol* 2004;335:209–219.
24. Zhang MY, Borges AR, Ptak RG, *et al.*: Potent and broad neutralizing activity of a single chain antibody fragment against cell-free and cell-associated HIV-1. *MAbs* 2010;2: 266–274.
25. Chen W, Zhu Z, Feng Y, Dimitrov DS: Human domain antibodies to conserved sterically restricted regions on gp120 as exceptionally potent cross-reactive HIV-1 neutralizers. *Proc Natl Acad Sci U S A* 2008;105:17121–17126.
26. Chen W, Dimitrov DS: Human monoclonal antibodies and engineered antibody domains as HIV-1 entry inhibitors. *Curr Opin HIV AIDS* 2009;4:112–117.
27. Chang TL, Chang CH, Simpson DA, *et al.*: Inhibition of HIV infectivity by a natural human isolate of *Lactobacillus jensenii* engineered to express functional two-domain CD4. *Proc Natl Acad Sci U S A* 2003;100:11672–11677.
28. Dennis MS, Zhang M, Meng YG, *et al.*: Albumin binding as a general strategy for improving the pharmacokinetics of proteins. *J Biol Chem* 2002;277:35035–35043.
29. Montefiori DC: *Evaluating Neutralizing Antibodies Against HIV, SIV, and SHIV in Luciferase Reporter Gene Assays*. John Wiley & Sons, Inc., New York, 2004.
30. Liu X, Lagenaur LA, Simpson DA, *et al.*: Engineered vaginal *Lactobacillus* strain for mucosal delivery of the human immunodeficiency virus inhibitor cyanovirin-N. *Antimicrob Agents Chemother* 2006;50:3250–3259.
31. Boskey ER, Cone RA, Whaley KJ, Moench TR: Origins of vaginal acidity: High D/L lactate ratio is consistent with bacteria being the primary source. *Hum Reprod* 2001;16: 1809–1813.
32. Boskey ER, Telsch KM, Whaley KJ, Moench TR, Cone RA: Acid production by vaginal flora in vitro is consistent with the rate and extent of vaginal acidification. *Infect Immun* 1999;67:5170–5175.
33. Hillier SL: The vaginal microbial ecosystem and resistance to HIV. *AIDS Res Hum Retroviruses* 1998;14(Suppl 1): S17–S21.
34. Beninati C, Oggioni MR, Boccanera M, *et al.*: Therapy of mucosal candidiasis by expression of an anti-idiotypic in human commensal bacteria. *Nat Biotechnol* 2000;18:1060–1064.
35. Oggioni MR, Beninati C, Boccanera M, *et al.*: Recombinant *Streptococcus gordonii* for mucosal delivery of a scFv microbicidal antibody. *Int Rev Immunol* 2001;20: 275–287.
36. Giomarelli B, Maggi T, Younson J, Kelly C, Pozzi G: Expression of a functional single-chain Fv antibody on the surface of *Streptococcus gordonii*. *Mol Biotechnol* 2004; 28:105–112.
37. Marcotte H, Koll-Klais P, Hultberg A, *et al.*: Expression of single-chain antibody against RgpA protease of *Porphyromonas gingivalis* in *Lactobacillus*. *J Appl Microbiol* 2006; 100:256–263.
38. Kruger C, Hu Y, Pan Q, *et al.*: In situ delivery of passive immunity by lactobacilli producing single-chain antibodies. *Nat Biotechnol* 2002;20:702–706.
39. Andersen KK, Marcotte H, Alvarez B, Boyaka PN, Hammarstrom L: In situ gastrointestinal protection against anthrax edema toxin by single-chain antibody fragment producing lactobacilli. *BMC Biotechnol* 2011;11:126.
40. Gunaydin G, Alvarez B, Lin Y, Hammarstrom L, Marcotte H: Co-expression of anti-rotavirus proteins (llama VHH antibody fragments) in *Lactobacillus*: Development and functionality of vectors containing two expression cassettes in tandem. *PLoS One* 2014;9:e96409.
41. Alvarez B, Krogh-Andersen K, Tellgren-Roth C, *et al.*: An exopolysaccharide-deficient mutant of *Lactobacillus rhamnosus* GG efficiently displays a protective Llama antibody fragment against rotavirus on its surface. *Appl Environ Microbiol* 2015;81:5784–5793.
42. Andersen KK, Strokappe NM, Hultberg A, *et al.*: Neutralization of *Clostridium difficile* toxin B mediated by engineered lactobacilli producing single domain antibodies. *Infect Immun* 2015;84:395–406.
43. Schnepf BC, Johnson PR: Adeno-associated virus delivery of broadly neutralizing antibodies. *Curr Opin HIV AIDS* 2014;9:250–256.

Address correspondence to:
 Laurel A. Lagenaur
 Osel, Inc.
 320 Logue Avenue
 Mountain View, CA 94043

E-mail: llagenaur@oselinc.com